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DIAGNOSTIC COMPOSITIONS, ELEMENTS, METHODS AND TEST KITS FOR AMPLIFICATION AND DETECTION OF HUMAN CMV DNA USING PRIMERS HAVING MATCHED MELTING TEMPERATURES

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DIAGNOSTIC COMPOSITIONS, ELEMENTS, METHODS AND TEST KITS FOR AMPLIFICATION AND DETECTION OF HUMAN CMV DNA USING PRIMERS HAVING MATCHED MELTING TEMPERATURES

Field of the Invention

This invention relates to diagnostic compositions, elements, methods and test kits for the amplification and detection of human cytomegaloviral (hCMV) DNA. In particular, it relates to improved methods of polymerase chain reaction (PCR) using test kits and buffered compositions containing "matched" primers for one or more target nucleic acids, one of which is hCMV DNA.

Background of the Invention

Cytomegalovirus is a double stranded DNA virus, approximately 240,000 nucleotides in length, and belonging to the Herpes virus group. Human cytomegalovirus has become increasingly of interest in a wide variety of disease states. In the United States, hCMV infects up to 1% of all newborns and results in significant morbidity and mortality in congenitally infected infants. Infections from hCMV also have a major impact on immunocompromised patients, such as those afflicted with acquired immunodeficiency syndrome (AIDS) and recipients of transplanted organs. People infected with the disease often suffer impairment of some of their vital organs, including the salivary glands, eyes, brain, kidneys and liver. In children, the hCMV infection represents the leading infectious cause of mental retardation and nonhereditary sensineural deafness in children. Furthermore, hCMV is associated with a wide variety of classical syndromes including mononucleosis and interstitial pneumonia. Studies suggest that hCMV is latent in white blood cells, and that it has an oncogenic potential and a possible association with

certain types of malignancy including Karposi's sarcoma.

Since hCMV is relatively common in people, a considerable effort has been made to isolate it and to diagnose disease in patients. Efforts to produce vaccines or to treat the disease continue unabated, as noted for example, in US-A-5,075,213 which describes radiolabeled probes for hCMV DNA.

Diagnosis of the presence of hCMV DNA by culture requires up to three weeks and is technically difficult and expensive. Immunological assays are not presently considered reliable. Earlier diagnosis could be important for treating immunosuppressed patients and for screening blood supplies. HCMV has a large linear double stranded DNA genome composed of two unique components as well as terminal repeat sequences.

Technology to detect minute quantities of nucleic acids (including hCMV DNA) has advanced rapidly over the last two decades including the development of highly sophisticated hybridization assays using probes in amplification techniques such as PCR. Researchers have readily recognized the value of such technology to detect diseases and genetic features in human or animal test specimens. The use of probes and primers in such technology is based upon the concept of complementarity, that is the bonding of two strands of a nucleic acid by hydrogen bonds between complementary nucleotides (also known as nucleotide pairs).

PCR is a significant advance in the art to allow detection of very small concentrations of a targeted nucleic acid. The details of PCR are described, for example, in US-A-4,683,195 (Mullis et al), US-A-4,683,202 (Mullis), and US-A-4,965,188 (Mullis et al) and by Mullis et al, *Methods of Enzymology*, 155, pp. 335-350 (1987), although there is

a rapidly expanding volume of literature in this field. Without going into extensive detail, PCR involves hybridizing primers to the strands of a targeted nucleic acid (considered "templates") in the presence of a polymerization agent (such as a DNA polymerase) and deoxyribonucleoside triphosphates under the appropriate conditions. The result is the formation of primer extension products along the templates, the products having added thereto nucleotides which are complementary to the templates.

Once the primer extension products are denatured, one copy of each template has been prepared, and the cycle of priming, extending and denaturation can be carried out as many times as desired to provide an exponential increase in the amount of nucleic acid which has the same sequence as the target nucleic acid. In effect, the target nucleic acid is duplicated (or "amplified") many times so that it is more easily detected. Despite the broad and rapid use of PCR in a variety of biological and diagnostic fields, there are still practical limitations which must be overcome to achieve the optimum success of the technology. PCR also produces considerable inefficiency in the use of expensive reagents.

It is well known that PCR is susceptible to a "carry-over" problem whereby amplified nucleic acids from one reaction may be inadvertently carried over into subsequent reactions using "fresh" PCR reaction mixtures, and thereby causing "false" positives when testing later specimens.

One approach to this problem is to completely contain the reagents for each PCR procedure so no reagents or by-products can be carried over into later reactions. Specially designed test packs or test devices have been designed to contain PCR procedures

for this reason. Such test packs are described, for example, in recently allowed U.S.S.N. 07/962,159 [filed October 15, 1992 by Schnipelsky et al as a continuation of U.S.S.N. 07/673,053 (filed March 21, 1991, now abandoned) which in turn is a CIP of U.S.S.N. 07/306,735 (filed February 3, 1989, now abandoned) now U.S. Patent 5,229,297]. These test devices are preferably, but not necessarily, used in PCR in combination with automatic PCR processing equipment such as that described in US-A-5,089,660 (Devaney Jr.) and in US-A-5,089,233 (Devaney Jr. et al). This equipment is characterized by its capability to simultaneously process several test specimens in separate test devices.

More preferably, it would be desirable to detect a multiplicity of target nucleic acids (or a multiplicity of nucleic acid sequences in the same nucleic acid) in a single test device. This is referred to herein as "multiplexing".

In one embodiment of PCR, a specific set of primers and a capture probe (a total of three oligonucleotides) are needed for each nucleic acid sequence which is to be amplified and detected. Thus, the three oligonucleotides are complementary and specific to that targeted nucleic acid sequence. For example, in multiplexing, if three nucleic acid sequences are to be amplified and detected, typically three sets of primers and probes are needed, one set specific for each sequence. Normally, detection of the multiple sequences requires a multiplicity of test devices, and perhaps different sets of PCR conditions (that is, temperature and time conditions).

It would be desirable, however, to amplify and detect a plurality of target nucleic acids simultaneously in the same test device and using "universal" processing equipment and conditions. This

cannot be done by merely selecting any set of primers and probes specific for each target nucleic acid from conventional sources. Where processing equipment is used to process several test devices simultaneously, or a single test device is designed for multiplexing, the equipment must be somehow adapted to provide optimum heating and cooling times and temperatures for each set of primers and probes, since they will all likely have individual optimum denaturaton temperatures. To adapt the equipment to randomly selected primers and probes in multiplexing would be a very expensive and cumbersome solution to the problem. Yet there is a great need for efficient, relatively inexpensive and rapid multiplexing to detect multiple nucleic acid sequences of hCMV DNA, or one or more nucleic acid sequences of hCMV DNA and one or more nucleic acid sequences of other target nucleic acids.

Summary of the Invention

The problems noted above are overcome by using, in PCR, an aqueous composition buffered to a pH of from about 7 to about 9, which comprises:

(a) first and second primers which are specific to and hybridizable with, respectively, first and second nucleic acid sequences which are in opposing strands of human cytomegaloviral DNA (hCMV DNA) and which are separated from each other along the opposing strands by from 90 to 400 nucleotides,

(b) third and fourth primers which are specific to and hybridizable with, respectively, third and fourth nucleic acid sequences which are in opposing strands of a second target DNA which is the same as or different from the first target DNA, the third and fourth nucleic acid sequences being different from the first and second nucleic acid sequences and being

separated from each other along the opposing strands by from 90 to 400 nucleotides,

each of the first, second, third and fourth primers having a T_m within the range of from about 65 to about 74°C, all of the primer T_m's being within about 5°C of each other, the first and second primers having nucleotide lengths which differ from each other by no more than 5 nucleotides, and the third and fourth primers having nucleotide lengths which differ from each other by no more than 5 nucleotides.

This invention also provides a diagnostic test kit for the amplification of hCMV DNA and a second target DNA comprising, in separate packaging:

a) the aqueous composition described above, and

b) at least one additional PCR reagent.

Further, a diagnostic test kit for the amplification of hCMV DNA and a second target DNA comprises, in separate packaging:

a) first and second primers which are specific to and hybridizable with, respectively, first and second nucleic acid sequences which are in opposing strands of human cytomegaloviral DNA (hCMV DNA) and which are separated from each other along the opposing strands by from 90 to 400 nucleotides, the first and second primers having the characteristics defined above,

b) third and fourth primers which are specific to and hybridizable with, respectively, third and fourth nucleic acid sequences which are in opposing strands of a second target DNA and which are separated from each other along the opposing strands by from 90 to 400 nucleotides, the third and fourth primers having the characteristics defined above, and

c) at least one additional PCR reagent.

Still further, this invention provides a method for the amplification and detection of human cytomegaloviral DNA and a second target DNA comprising:

A) simultaneously subjecting the denatured opposing strands of hCMV DNA and the denatured opposing strands of a second target DNA to polymerase chain reaction in the presence of:

i) the aqueous composition described above, and

ii) the additional PCR reagents: a thermostable DNA polymerase, a DNA polymerase cofactor and at least one dNTP, any or all of the additional PCR reagents being in the same or a different composition as defined in i),

to simultaneously amplify the opposing hCMV DNA strands and the opposing second target DNA strands, and

B) simultaneously detecting at least one of the amplified hCMV DNA strands and at least one of the amplified second target DNA strands as a simultaneous determination of the presence of hCMV DNA and the second target DNA.

The present invention provides an effective and efficient means for multiplexing, or amplifying and detecting a multiplicity of target nucleic acid sequences using the same test device, if desired, and the same processing equipment. It is particularly useful for the detection of one or more nucleic acid sequences of hCMV DNA alone and one or more nucleic acid sequences of one or more additional target nucleic acids. Any number of nucleic acid sequences can be amplified and determined simultaneously using the appropriate primer sets in combination.

These advantages are achieved by using a set of "matched" primers in PCR for each target nucleic acid, including a set for hCMV DNA. By "matched"

primers is meant primers having melting temperatures (T_m's) which are essentially the same, that is they differ by no more than about 5°C. Moreover, the T_m's of the two primers of each set are within the range of from about 65 to about 74°C, and the two primers have nucleotide lengths which differ from each other by no more than 5 nucleotides. Further, all of the primers of all primer sets used in the amplification method are also "matched", that is, they all have T_m's which differ by no more than about 5°C and all are within the range of from about 65 to about 74°C.

Brief Description of the Drawings

FIGS. 1-6 are sets of bar graphs showing dye signals for replicate PCR assays of various concentrations of both of hCMV DNA and HIV-I DNA, as described in Example 2 below.

FIGS. 7 and 8 are sets of bar graphs showing dye signals for replicate PCR assays of various concentrations of hCMV DNA, as described in Example 3 below.

Detailed Description of the Invention

The general principles and conditions for amplification and detection of nucleic acids using polymerase chain reaction are quite well known, the details of which are provided in numerous references including US-A-4,683,195, US-A-4,683,202, US-A-4,965,188, the disclosures of which are incorporated herein by reference. Thus, many details of PCR are not included herein. In view of the teaching in the art and the specific teaching provided herein, a worker skilled in the art should have no difficulty in practicing the present invention by making the adjustments taught herein to accomplish simultaneous amplification and detection of hCMV DNA and an additional target nucleic acid.

The present invention is directed to the amplification and detection of two or more specific target nucleic acids, one of which is hCMV DNA, present in a test specimen. Such specimens can include cellular or viral material, hair, body fluids or cellular materials containing DNA which can be detected.

Nucleic acids to be amplified and detected can be obtained from various sources including plasmids and naturally occurring DNA or RNA from any source (such as bacteria, yeast, viruses, plants and higher animals, humans). It may be extracted from various tissues including blood, peripheral blood mononuclear cells (PBMC), tissue material or other sources known in the art using known procedures. The present invention is particularly useful for the amplification and detection of one or more nucleic acid sequences in hCMV DNA and at least one other nucleic acid sequence found in mammalian DNA, bacterial DNA, fungal DNA, viral RNA, or DNA or RNA found in bacterial or virus-infected cells.

The method described herein can be used to amplify and detect target nucleic acids associated with infectious diseases, genetic disorders and cellular disorders such as cancers. It may also be used for forensic investigations and DNA typing. It is particularly useful for the detection of infectious agents, such as bacteria, viruses, fungi and protozoa, by detection of nucleic acids associated therewith.

Bacteria which can be detected include, but are not limited to, bacteria found in human blood, Salmonella species, Streptococcus species, Chlamydia species, Gonococcal species, *Mycobacterium tuberculosis*, *Mycobacterium fortuitum*, *Mycobacterium avium* complex, *Legionella pneumophila*, *Clostridium*

difficile, *Borrelia burgdorferei*, *Pneumocystis carinii*, *Mycoplasma Haemophilus influenzae*, Shigella species and Listeria species. Viruses which are detectable, besides cytomegalovirus, include, but are not limited to, herpes, Epstein Barr virus, influenza viruses, human papilloma virus, hepatitis and retroviruses such as HTLV-I, HTLV-II, HIV-I and HIV-II. Protozoan parasites, yeasts and molds are also detectable. Other detectable species would be readily apparent to one skilled in the art.

The invention is particularly useful for the simultaneous amplification and detection of one or more nucleic acid sequences of hCMV DNA with one or more nucleic acid sequences of a retroviral DNA (such as HIV-I, HIV-II or HTLV-I), *Mycobacterium tuberculosis* DNA, *Mycobacterium avium* DNA, Epstein Barr viral DNA, *Pneumocystis carinii* DNA, hepatitis DNA and respiratory syncytial viral DNA. It is particularly important to simultaneously amplify and detect hCMV DNA and DNA associated with infectious agents of similar pathogenicity, such as organisms which cause respiratory diseases, or HIV-I DNA.

A "target" DNA as used in this application also includes nucleic acids which are added to a test specimen to provide positive controls in the assays.

A "PCR reagent" refers to any of the reagents considered essential to PCR, namely primers for the target nucleic acid, a thermostable DNA polymerase, a DNA polymerase cofactor, and one or more deoxyribonucleoside-5'-triphosphates. Other optional reagents and materials used in PCR are described below.

The term "primer" refers to an oligonucleotide, whether naturally occurring or synthetically produced, which is capable of acting as a point of initiation of synthesis when placed under

conditions in which synthesis of a primer extension product complementary to a nucleic acid strand (that is, template) is induced. Such conditions include the presence of nucleotides (such as the four standard deoxyribonucleoside-5'-triphosphates), a thermostable DNA polymerase, and suitable temperature, pH and a DNA polymerase cofactor.

The primer is preferably single stranded for maximum efficiency in amplification, but can contain a double stranded region if desired. It must be long enough to prime the synthesis of extension products in the presence of the DNA polymerase. The exact size of each primer will vary depending upon the use contemplated, the complexity of the targeted sequence, reaction temperature and the source of the primer. Generally, the primers used in this invention will have from 12 to 60 nucleotides, and preferably, they have from 20 to 35 nucleotides. More preferably, each primer in a set has from 20 to 30 nucleotides. The lengths of the primers in each primer set differ from each other by no more than 5 nucleotides, and preferably by no more than 2 nucleotides. Most preferably, the primers of each set have the same length.

One set of primers used in the practice of the invention includes first and second primers which are specific to, respectively, first and second nucleic acid sequences in opposing strands of hCMV DNA. The first and second sequences are spaced along the opposing strands from each other by from 90 to 400 nucleotides, and preferably from 100 to 300 nucleotides, with a spacing of from 110 to 275 being most preferred. Thus, the two primers hybridize to nucleic acid sequences which are relatively close to each other along the opposing strands.

A second set of primers (including third and fourth primers) can be used to detect third and fourth sequences of opposing strands of hCMV DNA (different from the first and second nucleic acid sequences of hCMV DNA) or of a target DNA from a different source.

For every set of primers used in this invention, the primers in the set have a T_m within the range of from about 65 to about 74°C, preferably within the range of from about 67 to about 74°C. In addition, the primer T_m's are within about 5°C of each other, and preferably they differ by no more than 2°C. Further still, the T_m's of the primers in each additional set differ from the T_m's of all other primers in the other sets of primers by no more than about 5°C, and preferably by no more than about 2°C. The third and fourth primers (and the primers in any additional primer set) also hybridize to nucleic acid sequences in the opposing strands of the particular target nucleic acid, which sequences are spaced apart along the strands by from 90 to 400 nucleotides (more preferably, from 100 to 300, and most preferably from 110 to 275 nucleotides apart).

These characteristics and relationships among all of the primers allow effective and efficient multiplexing using a single test device and the same PCR processing equipment and conditions.

T_m (melting temperature) is defined herein as the temperature at which about one-half of a double-stranded DNA molecule is denatured. The determination of T_m can be accomplished using several standard procedures, based on ultraviolet hypochromism, for example, by monitoring the spectrum at 260 nm as described in Biochemistry- The Molecular Basis of Cell Structure and Function, 2nd Edition, Lehninger, Worth Publishers, Inc., 1970, pp. 876-7. The various methods

of determining T_m values may produce slightly differing values for the same DNA molecule, but those values should not vary from each other by more than about 2 or 3°C.

Preferably, the T_m values described herein for the specific primers and probes are calculated using the formula:

$$T_m\ (°C) = 67.5 + 0.34(\%\ G + C) - 395/N$$

wherein "G" and "C" represent the number of guanine and cytosine nucleotides, respectively, and "N" represents the total number of nucleotides in the primer or probe. T_m values obtained by this calculation correlate very well with the values determined empirically at room temperature using conventional UV hypochromism and a conventional Hewlett-Packard diode array spectrophotometer (scanning rate of about +1°C/min.) for a solution of oligonucleotide (primer or capture probe) in 10 mmolar tris(hydroxymethyl)-aminomethane buffer (pH 8.5) having an ionic strength of at least about 60 mmolar provided by one or more inorganic or organic salts, such as magnesium chloride, magnesium sulfate, potassium chloride, sodium chloride, and others readily apparent to one skilled in the art. The amount of oligonucleotide and its complement in the solution used to determine the noted formula was sufficient to provide an optical density of from about 0.5 to about 1.0 OD units.

The primers used in the present invention are selected to be "substantially complementary" to the specific nucleic acid sequences to be primed and amplified. This means that they must be sufficiently complementary to hybridize with the respective nucleic acid sequences to form the desired hybridized products and then be extendable by a DNA polymerase. In the preferred and most practical situation, the primers

have exact complementarity to the nucleic acid sequences of interest.

Primers of particular usefulness in the amplification and detection of hCMV DNA include, but are not limited to, those having the sequences in the three primer sets shown below with the T_m in parenthesis:

Primer set 1:

SEQ ID NO:1: 5'-GAGGCTATTG TAGCCTACAC TTTGG-3'(68.4°C)

SEQ ID NO:2: 5'-CAGCACCATC CTCCTCTTCC TCTGG-3'(72.7°C),

Primer set 2:

SEQ ID NO:3: 5'-TGCACTGCCA GGTGCTTCGG CTCAT-3'(72.7°C)

SEQ ID NO:4: 5'-CACCACGCAG CGGCCCTTGA TGTTT-3'(72.7°C), and

Primer set 3:

SEQ ID NO:16 5'-CATTCCCACT GACTTTCTGA CGCACGT-3' (70.9°C)

SEQ ID NO:17 5'-TGAGGTCGTG GAACTTGATG GCGT-3' (70.0°C).

The primers of Set 1 are complementary to nucleic acid sequences in the "major immediate early" region of hCMV DNA, and the primers in Set 2 are complementary to nucleic acid sequences in the "late antigen" region of hCMV DNA. The primers in Set 3 are complementary to nucleic acid sequences in the "major capsid protein" region of hCMV DNA. The primers in Primer Set 2 are preferred in the practice of this invention. The primers noted above are not limited in use to the particular set, but can be used with any primer for hCMV DNA which has the properties noted herein.

Primers useful for the amplification and detection of additional target nucleic acids would be readily determinable by a skilled worker in the art by consultation with the considerable literature in this field to determine appropriate nucleic acid sequences of target nucleic acids. Those sequences can then be used as patterns for preparing primers using known technology. The primers can be readily screened by determining if they have the requisite T_m (using appropriate methods defined above) and other requirements as defined above.

Primers useful herein can be obtained from a number of sources or prepared using known techniques and equipment, including for example, an ABI DNA Synthesizer (available from Applied Biosystems) or a Biosearch 8600 Series or 8800 Series Synthesizer (available from Milligen-Biosearch, Inc.). Procedures for using this equipment are well known and described for example in US-A-4,965,188, incorporated herein by reference. Naturally occurring primers isolated from biological sources are also useful (such as restriction endonuclease digests).

As used herein, a "probe" is an oligonucleotide which is substantially complementary to a nucleic acid sequence of the target nucleic acid (hCMV DNA or any additional target nucleic acid) and which is used for detection or capture of the amplified target nucleic acid. The probes generally have from 10 to 40 nucleotides, and a T_m greater than about 50°C. Moreover, the probes are hybridizable with a nucleic acid sequence of the particular target nucleic acid at a temperature in the range of from about 45 to about 55°C. In the use of a multiplicity of probes for capturing a multiplicity of amplified target nucleic acids in the practice of this invention, all of the

capture probes have T_m's which differ by no more than about 15°C. Preferably, the capture probe T_m's used simultaneously differ by no more than about 5°C.

Representative capture probes for hCMV DNA include, but are not limited to, the following oligonucleotides, with the T_m in parenthesis:

SEQ ID:NO:5 5'-GGTGTCACCC CCAGAGTCCC CTGTACCCGC-3' (78.9°C),

SEQ ID:NO:6 5'-GACACAGTGT CCTCCCGCTC CTCCTGAGCA-3' (76.5°C),

SEQ ID:NO:7 5'-GTGGAAGGCG GCTCGCTGGA AGCCGGTCGT-3' (78.9°C),

SEQ ID:NO:8 5'-GAACCGAGGG CCGGCTCACC TCTATGTTGG-3' (76.5°C), and

SEQ ID:NO:18 5'-GGTCATCGCC GTAGTAGATG CGTAAGGCCT-3' (74.1°C).

The first two listed probes are complementary to nucleic acid sequences in the "major immediate early" region of hCMV DNA, the next two listed probes are complementary to nucleic acid sequences in the "late antigen" region of hCMV DNA, and the last probe is complementary to a nucleic acid sequence in the "major capsid protein" region of hCMV DNA. The probe identified as SEQ ID:NO:8 is preferred in the practice of this invention.

Probes useful for the detection or capture of additional target nucleic acids would be readily apparent to one skilled in the art once the targeted nucleic acid sequences are known. Such sequences are known in the literature. Thus, the practice of this invention is adequately enabled by knowing those sequences and following the teaching herein regarding the amplification and detection of hCMV DNA. Presently unknown target nucleic acids will also be similarly amplified and detected because this technology could

predictably be used in a similar fashion. Potential probes can be screened to see if they have the requisite T_m as defined above. Such probes can be prepared using the same procedures and starting reagents described for primers above.

Additional PCR reagents necessary for PCR include a thermostable DNA polymerase, a DNA polymerase cofactor and appropriate dNTP's. These reagents can be provided individually, as part of a test kit, in reagent chambers of a test device, or in the composition of this invention.

A thermostable DNA polymerase is an enzyme which will add deoxynucleoside monophosphate molecules to the 3' hydroxy end of the primer in a complex of primer and template, but this addition is in a template dependent manner (that is, dependent upon the specific nucleotides in the template). Synthesis of extension products proceeds in the 5' to 3' direction of the newly synthesized strand (or in the 3' to 5' direction of the template) until synthesis is terminated.

The DNA polymerase is "thermostable" meaning that it is stable to heat and preferentially active at higher temperatures, especially the high temperatures used for denaturation of DNA strands. More particularly, the thermostable DNA polymerases are not substantially inactivated at the high temperatures used in polymerase chain reactions as described herein. Such temperatures will vary depending upon a number of reaction conditions, including pH, the nucleotide composition of the target nucleic acid and primers, the length of primer, salt concentration and other conditions known in the art and will be in the ranges noted below.

A number of thermostable DNA polymerases have been reported in the art, including those mentioned in

detail in US-A-4,965,188 and US-A-4,889,818 (Gelfand et al), both incorporated herein by reference. Particularly useful polymerases are those obtained from various Thermus bacterial species, such as *Thermus aquaticus, Thermus thermophilus, Thermus filiformis* or *Thermus flavus*. Other useful thermostable polymerases are obtained from a variety of other microbial sources including *Thermococcus literalis, Pyrococcus furiosus,* Thermotoga sp. and those described in WO-A-89/06691 (published July 27, 1989). Some useful polymerases are commercially available. A number of techniques are known for isolating naturally-occurring polymerases from organisms, and for producing genetically engineered enzymes using recombinant techniques, as noted in the art cited in this paragraph and as described in EP-A-0 482 714 (published April 29, 1992).

A DNA polymerase cofactor refers to a nonprotein compound on which the enzyme depends for activity. Thus, the enzyme is catalytically inactive without the presence of the cofactor. A number of such materials are known cofactors including manganese and magnesium compounds. Such compounds contain the manganese or magnesium in such a form that divalent cations are released into an aqueous solution. Useful cofactors include, but are not limited to, manganese and magnesium salts, such as chlorides, sulfates, acetates and fatty acid salts (for example, butyric, caproic, caprylic, capric and lauric acid salts). The smaller salts, that is chlorides, sulfates and acetates, are preferred.

Magnesium salts, such as magnesium chlorides and sulfates are most preferred in the practice of the invention.

Also needed for PCR is a deoxyribonucleoside-5'-triphosphate, such as dATP, dCTP, dGTP, dTTP or

dUTP. Analogues such as dITP and 7-deaza-dGTP are also useful. It is conventional to identify the preferred materials, dATP, dCTP, dGTP and dTTP, collectively as dNTP's.

The PCR reagents described herein are provided and used in PCR in any concentration suitable for a given process. The minimal amounts of primers, thermostable DNA polymerase, cofactors and deoxyribonucleotide-5'-triphosphates needed for amplification and suitable ranges of each are well known in the art. Preferably, from about 0.1 to about 50 units of thermostable DNA polymerase per 100 µl of reaction mixture are used for PCR, depending upon the particular activity of a given enzyme. More preferably, from about 10 to about 25 units of DNA polymerase/100 µl of solution are used. A "unit" is defined herein as the amount of enzyme activity required to incorporate 10 nmoles of total nucleotides (dNTP's) into an extending nucleic acid chain in 30 minutes at 74°C. The amount of each primer is at least about 0.075 µmolar with from about 0.1 to about 2 µmolar being preferred, but other amounts are well known in the art. The cofactor is generally present in an amount of from about 2 to about 15 mmolar. The amount of each dNTP is from about 0.25 to about 3.5 mmolar (about 1 to about 14 mmolar for a total of four common dNTP's).

The aqueous composition of this invention is buffered to a pH of from about 7 to about 9 (preferably from about 8 to about 8.5) using one or more suitable buffers including, but not limited to, tris(hydroxy-methyl)aminomethane and others readily apparent to one skilled in the art.

A particularly useful composition of this invention is a buffered mixture of the primers noted

herein, a magnesium cofactor as noted above, each of dATP, dCTP, dGTP and dTTP as noted above, gelatin or a similar hydrophilic colloidal material (in an amount of at least about 5%, by weight), and an alkali metal salt (such as sodium chloride or potassium chloride) present in an amount of from about 10 to about 100 mmolar. More preferably, this composition also includes an appropriate amount of a thermostable DNA polymerase (as described above), and a monoclonal antibody to such DNA polymerase, which antibody inhibits its enzymatic activity at temperatures below about 50°C, but which antibody is deactivated at higher temperatures. Representative monoclonal antibodies are described in U.S.S.N. 07/958,144 (filed October 7, 1992 by Scalice et al). Two such monoclonal antibodies are readily obtained by a skilled artisan using conventional procedures, and starting materials including either of hybridoma cell lines HB 11126 and 11127 deposited with the American Type Culture Collection (Rockville, Maryland). The monoclonal antibody is present in an amount of from about 5:1 to 500:1 molar ratio to the DNA polymerase.

A preferred composition of this invention is shown in Example 1 below.

A target nucleic acid (including hCMV DNA) can be obtained from any of a variety of sources as noted above, such as a whole blood sample. Generally, it is extracted in some manner to make it available for contact with the primers and other PCR reagents. This usually means removing unwanted proteins and cellular matter from the specimen in a suitable manner. Various procedures are known in the art, including those described by Laure et al in *The Lancet*, pp. 538-540 (Sept. 3, 1988), Maniatis et al, Molecular Cloning: A

Laboratory Manual, pp. 280-281 (1982), Gross-Belland et al in *Eur.J.Biochem.*, 36, 32 (1973) and US-A-4,965,188. Extraction of DNA from whole blood or components thereof are described, for example, in EP-A-0 393 744 (published October 24, 1990), Bell et al, *Proc. Natl. Acad. Sci. USA*, 78(9), pp. 5759-5763 (1981) and Saiki et al, *Bio/Technology*, 3, pp. 1008-1012 (1985). One preferred method for extraction is described below in relation to the examples.

Since the nucleic acid to be amplified and detected is usually in double stranded form, the two strands must be separated (that is, denatured) before priming can take place. This can occur during the extraction process, or be a separate step afterwards. Denaturation is accomplished using a heat treatment alone or in combination with any suitable other physical, chemical or enzymatic means as described in the art. Initial denaturation is generally carried out by heating the specimen suspected of containing the targeted nucleic acid at a first temperature of from about 85 to about 100°C for a suitable time, for example from about 1 second to 3 minutes.

The denatured strands are then cooled to a temperature which is generally in the range of from about 55 to about 70°C for priming the strands. The time needed for cooling the denatured strands will vary depending upon the type of apparatus used for the PCR process.

Once the denatured strands are cooled to the second temperature, the reaction mixture containing PCR reagents is incubated at a suitable temperature to effect formation of primer extension products. Generally, this temperature is at least about 50°C, and preferably in the range of from about 62 to about 75°C. The time for incubation can vary widely depending upon

the incubation temperature and the length of extension products desired, but in preferred embodiments, it is from about 1 to about 120 seconds. Each cycle of PCR can be carried out using either two or three different temperatures, one for denaturation, and a second or third temperature for priming and/or primer extension product formation. That is, some PCR processes utilize a second temperature for priming, and a third temperature for primer extension. Preferably, the same temperature (within the range of from about 62 to about 75°C) is used for both priming and primer extension.

If the hybridized primer extension products are then denatured, PCR can be carried out further in as many cycles of priming, extension and denaturation as desired. Generally, at least 20 cycles will be carried out, with from 20 to 50 cycles being preferred.

The amplification method of this invention is preferably conducted in a continuous, automated manner so that the reaction mixture is temperature cycled in a controlled manner for desired preset times. A number of instruments have been developed for this purpose, as one of ordinary skill in the art would know.

One such instrument for this purpose is described in some detail in US-A-4,965,188 and EP-A-0 236 069. Generally, this instrument includes a heat conducting container for holding a number of reaction tubes containing reaction mixture, a means for heating, cooling and temperature maintenance, and a computing means to generate signals to control the amplification sequence, changes in temperature and timing.

A preferred instrument for processing amplification reactions in a disposable chemical test pack is described in some detail in US-A-5,089,233 (Devaney et al), incorporated herein by reference. In general, this instrument comprises a surface for

supporting one or more chemical test packs, pressure applicators supported above the surface for acting on the reaction pack to transfer fluids between adjacent chambers in the test pack, and means for operating the pressure applicators through a range of movements extending across the test pack.

EP-A-0 402 994 provides details of useful chemical test packs which can be processed using the instrument described in US-A-5,089,233 (noted above). Also described therein are means for heating and cooling the test pack at repeated intervals (that is, through cycles) appropriate for the method of the present invention.

Further details regarding useful PCR processing equipment can be obtained from the considerable literature in this field, and would be readily ascertained by one skilled in the art.

It is also useful for the method of this invention to be carried out in a suitable container. The most crude container would be a test tube, cuvette, flask or beaker, but more sophisticated containers have been fashioned in order to facilitate automated procedures for performing the method (see for example, WO-A-91/12342). For example, cuvette and chemical test packs (also known as pouches), constructed to provide certain temperature characteristics during the practice of the method, are described in US-A-4,902,624 (Columbus et al), US-A-5,173,260 (Zander et al) and recently allowed U.S.S.N. 07/962,159 (noted above), all incorporated herein by reference. Such test packs have a multiplicity of reagent chambers having various reagents, buffers and other materials which are useful at various stages in the amplification or detection method. The aqueous composition of this invention can be incorporated into a reaction chamber for use in PCR.

The packs can be appropriately and rapidly heated and cooled in cycles to promote the various steps of the amplification method of this invention. Other useful containers could be suitably fashioned for automated or single use of the method of this invention.

Detection of the amplified hCMV DNA (and any additional amplified target nucleic acid) can be accomplished in a number of known ways, such as those described in US-A-4,965,188 (noted above). For example, it can be detected using Southern blotting or dot blot techniques. Alternatively, amplification can be carried out using primers that are appropriately labeled (such as with a radioisotope), and the amplified primer extension products are detected using procedures and equipment for detection of radioisotopic emissions.

In one embodiment, the amplified target nucleic acid is detected using an oligonucleotide probe which is labeled for detection and can be directly or indirectly hybridized with one of the primer extension products. Procedures for attaching labels and preparing probes are well known in the art, for example, as described by Agrawal et al, *Nucleic Acid Res.*, 14, pp. 6227-45 (1986), US-A-4,914,210 (Levenson et al) relating to biotin labels, US-A-4,962,029 (Levenson et al) relating to enzyme labels, and the references noted therein. Useful labels include radioisotopes, electron-dense reagents, chromogens, fluorogens, phosphorescent moieties, ferritin and other magnetic particles (see US-A-4,795,698 issued to Owen et al and US-A-4,920,061 issued to Poynton et al), chemiluminescent moieties and enzymes (which are preferred). Useful enzymes include, glucose oxidase,

peroxidases, uricase, alkaline phosphatase and others known in the art and can be attached to oligonucleotides using known procedures. Substrate reagents which provide a chemiluminescent or colorimetric signal in the presence of a particular enzyme label would be readily apparent to one skilled in the art.

Where the label is a preferred enzyme such as a peroxidase, at some point in the assay, hydrogen peroxide and a suitable dye-forming composition are added to provide a detectable dye (that is, a colorimetric signal). For example, useful dye-providing reagents include tetramethylbenzidine and derivatives thereof, and leuco dyes, such as triarylimidazole leuco dyes (as described in US-A-4,089,747 of Bruschi), or other compounds which react to provide a dye in the presence of peroxidase and an oxidant such as hydrogen peroxide. Particularly useful dye-providing compositions are described in US-A-5,024,935 (McClune et al), incorporated herein by reference. Chemiluminescent signals can be generated using acridinium salts or luminol and similar compounds in combination with enhancers in the presence of peroxidase.

Detection of the presence of the probe which is in the complementary product can be achieved using suitable detection equipment and procedures which are well known. Certain probes may be visible to the eye without the use of detection equipment.

In a preferred embodiment, one or both of the primers in each primer set used to detect a target nucleic acid is labeled with a specific binding moiety. The specific binding moiety can

be the same or different for each set of primers. Such labels include any molecule for which there is a receptor molecule that reacts specifically with the specific binding moiety. Examples of specific binding pairs (one of which can be the label) include, but are not limited to, avidin/biotin, sugar/lectin, streptavidin/biotin, antibody/hapten, antibody/antigen and others readily apparent to one skilled in the art. The receptor is then conjugated with a detectable label moiety, such as an enzyme using known technology.

Most preferably, one or both primers are labeled with biotin (or a equivalent derivative thereof), and the amplified target nucleic acid is detected using a conjugate of avidin (or streptavidin) with an enzyme. The enzyme attached to the specific binding complex is then detected using the appropriate substrate reagents.

In order for the amplified target nucleic acid to be detected, it is often useful (but not necessary) for it to be separated from the other materials in the reaction medium. This is done by any of a number of ways, including using a capture probe which is covalently attached to a water-insoluble support. The capture probe hybridizes with the amplified target nucleic acid and the captured material can then be separated from unhybridized materials in a suitable manner, such as by filtration, centrifugation, washing or other suitable separation techniques.

Capture probes can be attached to water-insoluble supports using known attachment techniques. One such technique is described in EP-A-0 439 222 (published September 18, 1991).

Other techniques are described for example in US-A-4,713,326 (Dattagupta et al), US-A-4,914,210 (Levenson et al) and EP-B-0 070 687 (published January 26, 1983). Useful separation means are microporous filtration membranes such as the polyamide membranes marketed by Pall Corp. (for example as LOPRODYNE™ or BIODYNE™ membranes) which can be used to separate captured target nucleic acids from unhybridized materials.

Any useful solid support can be used for separation of water-insoluble product for detection, including a microtiter plate, test tube, beaker, beads, film, membrane filters, filter papers, gels, magnetic particles or glass wool. It can be made of a number of materials including glass, ceramics, metals, naturally occurring or synthetic polymers, cellulosic materials, filter materials and others readily apparent to one of ordinary skill in the art. Particularly useful solid support materials are polymeric or magnetic particles generally having an average particle size of from about 0.001 to about 10 mmeters. Further details about such preferred polymeric particles, including useful monomers, methods of preparing them and attachment of receptor molecules, are provided in US-A-4,997,772 (Sutton et al), US-A-5,147,777 (Sutton et al), US-A-5,155,166 (Danielson et al), .

The detection can also be carried out by immobilizing a capture probe on a flat substrate, such as the microporous filtration membranes described above, or on thin polymeric films, uncoated papers or polymer coated papers, a number of which are known in the art. Other details about such materials are provided in U.S.S.N.

07/571,560 (filed September 4, 1990 as a CIP of U.S.S.N. 07/306,954, filed February 3, 1989 by Findlay et al, and corresponding to EP-A-0 408 738, published January 23, 1991).

Particularly useful arrangements of a capture reagent are described, for example, in U.S.S.N. 07/837,772 (filed February 18, 1992 by Sutton et al, corresponding to WO 92/16659, published October 1, 1992) and US-A-5,173,260 (noted above). The capture probes are covalently attached (either directly or through chemical linking groups) to the same type of polymeric particles, and the resulting capture reagents are immobilized on a heat or ultrasonic sealable support (for example, a sheet, membrane, fibrous mat, film). One particularly useful sealable support is a laminate of polyethylene and a polyester such as polyethylene terephthalate. The capture reagents can be disposed in distinct regions on the water-insoluble support which is part of a suitable test device (as described above). Such test devices can also be defined as diagnostic elements. For example, the support can have disposed thereon a plurality of stripes or spots of various capture reagents. The multiplicity of capture probes arranged in defined regions on such supports all have the T_m values as described above, that is the T_m values differ by no more than about 15°C.

Thus, according to one embodiment of this invention, a diagnostic element comprises a water-insoluble, heat or ultrasonic sealable support, having disposed thereon in distinct regions thereof, a plurality (two or more) of capture reagents,

each of the capture reagents having a capture probe specific for and hybridizable with a distinct (that is, unique to that capture probe) target DNA at a temperature of from about 40 to about 55°C (preferably from about 45 to about 53°C), each of the capture probes having from 10 to 40 nucleotides and a T_m greater than about 50°C, and the T_m's of all capture probes differing by no more than about 15°C (preferably no more than about 5°C),

at least one of the capture probes being specific for and hybridizable with hCMV DNA.

The present invention includes diagnostic test kits which can include the composition of this invention, an additional PCR reagent and other materials, equipment and instructions needed to carry out the method of the invention. The kits can include one or more detection or capture probes, multiple primer sets and test devices for the assays. In some embodiments, the kit components are separately packaged for use in a suitable container or test device. In other embodiments, the kit contains a test device having within separate reagent chambers or compartments, some or all of the reagents and compositions needed for the assay. In such embodiments, the separate packaging of the kit components can be within a single test device.

The following examples are included to illustrate the practice of this invention, and are not meant to be limiting in any way. All percentages are by weight unless otherwise noted.

Materials and Methods for Examples:

Recombinant DNA polymerase from *Thermus aquaticus* was prepared as described in U.S.S.N. 602,848

(filed October 26, 1990 by Sullivan) and had an activity of about 250,000 units/mg of protein.

The primers and probes were prepared using known starting materials and procedures using an Applied Biosystems Model 380B, three column DNA synthesizer using standard phosphoramidite chemistry and the ABI 1 mmolar scale, fast cycle protocol. Nucleoside-3'-phosphoramidites and nucleoside derivitized controlled pore glass supports were obtained from Applied Biosystems. The primers had the sequences identified above. They were functionalized at the 5' end with two tetraethylene glycol spacers followed by a single commercially available DuPont biotin phosphoramidite. The probes were functionalized at the 3' end with two tetraethylene glycol spacers followed by a single aminodiol linking group according to US-A-4,914,210 (noted above). All purifications were carried out using a nucleic acid purification column, followed by reversed phase HPLC techniques.

The novel oligonucleotides of this invention having the sequences:

SEQ ID NO:3: 5'-TGCACTGCCA GGTGCTTCGG CTCAT-3',
SEQ ID NO:16 5'-CATTCCCACT GACTTTCTGA CGCACGT-3',
SEQ ID NO:17 5'-TGAGGTCGTG GAACTTGATG GCGT-3',
SEQ ID:NO:18 5'-GGTCATCGCC GTAGTAGATG CGTAAGGCCT-3',
SEQ ID:NO:19 5'-GGAATGACGC AAGGACATAT GGGCGT-3',
SEQ ID:NO:20 5'-CCCAGGTGCA CACCAATGTG GTGGAT-3',
SEQ ID:NO:21 5'-GGACTGTGCG CGTTGTATAC CCTGC-3',
SEQ ID:NO:22 5'-ACTCCCGAAG CGAATGGCAC GTGGA-3',
SEQ ID:NO:23 5'-CATAGCTTGT GCCCGTGTGG CACGT-3',
SEQ ID:NO:24 5'-CCAAGACGAG ACCGTCAGAG CTGGT-3',
SEQ ID:NO:25 5'-AAGCTGTTGC CGCCATCAAA TAAACG-3', and
SEQ ID:NO:26 5'-CTGCGTTAGA CCGAGAACTG TGGATAAAGG-3'

were prepared using the procedures just described.

Deoxyribonucleotides (dNTP's) were obtained from Sigma Chemical Co.

Positive controls can be used in the PCR assays of this invention by incorporating into the assay specimen, another DNA which are considered a positive control target DNA. Primers and probes specific to the positive control target DNA are then used to amplify and detect the positive control target DNA using the same procedures, reagents and equipment used for the unknown target DNA's. The primers and probes used in this manner are also "matched" as described herein. The primers and probes of these control sets can be used in positive control assays in combination with the detection of any target DNA, not just hCMV DNA.

Primers useful for amplifying positive control target DNA's include the following primer sets (with T_m values):

Positive control primer set 1:

SEQ ID:NO:19 5'-GGAATGACGC AAGGACATAT GGGCGT-3' (72.5°C)

SEQ ID:NO:20 5'-CCCAGGTGCA CACCAATGTG GTGGAT-3' (71.1°C),

Positive control primer set 2:

SEQ ID:NO:21 5'-GGACTGTGCG CGTTGTATAC CCTGC-3' (72.7°C)

SEQ ID:NO:22 5'-ACTCCCGAAG CGAATGGCAC GTGGA-3' (72.7°C),

Positive control primer set 3:

SEQ ID:NO:23 5'-CATAGCTTGT GCCCGTGTGG CACGT-3' (72.7°C),

SEQ ID:NO:24 5'-CCAAGACGAG ACCGTCAGAG CTGGT-3' (72.7°C).

The primers of positive control primer set 1 is specific to and hybridizable with nucleic acid sequences in opposing strands of avian endogenous provirus ev-1 DNA. The primers in the other two control primer sets are specific to and hybridizable with a synthetically prepared oligonucleotide used as a positive control "target" DNA.

Capture probes which can be used with the three primer sets identified above are as follows (with T_m values):

SEQ ID:NO:25 5'-AAGCTGTTGC CGCCATCAAA TAAACG-3' (67.1°C), and

SEQ ID:NO:26 5'-CTGCGTTAGA CCGAGAACTG TGGATAAAGG-3' (71.7°C).

The capture probe identified as SEQ ID:NO:25 can be used with positive control primer set 1, while SEQ ID:NO:26 can be used with either positive control primer set 2 or 3.

The monoclonal antibody specific to the noted DNA polymerase was prepared as described in U.S.S.N. 07/958,144 (filed October 7, 1992 by Scalice et al). Generally, it was prepared from the immune cells of DNA polymerase immunized mice using conventional procedures, such as those described by Milstein et al, *Nature* 256, pp. 495-497, 1975 and hybridoma cell lines (either HB 11126 or 11127 from ATCC), whereby antibody secreting cells of the host animal were isolated from lymphoid tissue (such as the spleen) and fused with SP2/0-Ag14 murine myeloma cells in the presence of polyethylene glycol, diluted into selective media and plated in multiwell tissue culture dishes. About 7-14 days later, the hybridoma cells containing the antibodies were harvested, and purified using conventional techniques.

An avidin-peroxidase conjugate solution comprised a commercially available (Zymed Laboratories, Inc.) conjugate of avidin and horseradish peroxidase (126 µl/l), casein (0.5%) and merthiolate (0.5%).

A wash solution (pH 7.4) contained sodium phosphate, monobasic 1-hydrate (25 mmolar), sodium chloride (373 mmolar), (ethylenedinitrilo)tetracetic acid disodium salt (2.5 mmolar), ethylmercurithiosalicylic acid sodium salt (25 µmolar), and decyl sodium sulfate (38 mmolar).

The dye-providing composition (pH 6.8) contained 4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxy-3-methoxyphenyl)imidazole (250 µmolar), poly(vinyl pyrrolidone) (112 mmolar), agarose (0.5%), diethylenetriaminepentaacetic acid (100 mmolar), 4'-hydroxyacetanilide (5 mmolar) and sodium phosphate, monobasic, 1-hydrate (10 mmolar).

Pure hCMV target DNA was obtained by purifying commercially available crude hCMV DNA (Advanced Biotech's strain AD169) using a conventional sucrose gradient and phenol/chloroform extraction procedures. The concentration of hCMV DNA was then determined at A_{260} and target dilutions of varying calculations of copy number were made for experimental use in TE buffer [tris(hydroxymethyl)aminomethane (1 mmolar), ethylenediaminetetraacetic acid (0.1 mmolar)]. A sample (10 µl) of the diluted solutions were added to 300 µl of PCR reaction mixture.

Two "nonsense" probes were used as controls. One was SEQ ID:NO:5 since only a nucleic acid sequences from the "late antigen" region was amplified in the following examples, and the other had the sequence:

SEQ ID:NO:9 5'-ATCCTGGGAT TAAATAAAAT AGTAAGAATG ~~TATAGCCCTA~~ TATAGCCCTA C-3'

Capture reagents were prepared by attaching the capture probes identified above to particles of poly[styrene-co-3-(p-vinylbenzylthio)propionic acid] (95:5 molar ratio, 1 mm average diameter) in the following manner. A suspension of the particles in water was washed twice with 2-(N-morpholino)ethanesulfonic acid buffer (0.1 molar, pH 6), and suspended to approximately 10% solids. A sample (3.3 ml) of the washed particles, diluted to 3.33% solids in the buffer (0.1 molar), was mixed with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.1 ml of 84 mg/ml water) and the appropriate probe (983 µl of 44.44 OD/ml nanopure water). The resulting suspension was heated at 50°C in a water bath for about two hours with intermittent mixing and centrifuged. The particles were washed three times with tris(hydroxymethyl)aminomethane buffer (0.01 molar, pH 8) containing (ethylenedinitrilo)-tetraacetic acid disodium salt (0.0001 molar) and resuspended therein to 4% solids. The capture probe used to detect hCMV DNA ("late antigen" region) was SEQ ID:NO:8 identified above.

Control capture reagents were similarly prepared using the "nonsense" probes identified above.

All of the capture reagents were mounted on a heat-sealable polyethylene/polyester laminate (treated by corona discharge) in test devices prepared as described in WO-A-92/16659 (noted above) so that the assay fluids and reagents contacted all of the capture reagents at about the same time. PCR was carried out using an automated Kodak PCR processor which is described in detail in US-A-5,089,233 (noted above), which is incorporated herein by reference.

Primers (and T_m) used for the amplification and detection of HIV-I DNA were as follows:

SEQ ID:NO:10 5'-AGTGGGGGGA CATCAAGCAG CCATGCAA-3' (73.4°C),

SEQ ID:NO:11 5'-CTTGGTTCTC TCATCTGGCC TGGTGC-3' (72.1°C),

SEQ ID:NO:12 5'-TAGCACCCAC CAGGGCAAAG AGAAGAGT-3' (72.1°C), and

SEQ ID:NO:13 5'-AGATGCTGTT GCGCCTCAAT AGCCCTGA-3' (72.1°C).

SEQ ID:NO:10 and SEQ ID:NO:11 are primers for the "gag" region of HIV-I DNA, and SEQ ID:NO:12 and SEQ ID:NO:13 are primers for the "env" region of HIV-I DNA.

Capture probes (and T_m's) used for the detection of amplified HIV-I DNA were as follows, with the first one being for the "gag" region of HIV-I DNA and the second one for the "env" region of HIV-I DNA:

SEQ ID:NO:14 5'-GAGACCATCA ATGAGGAAGC TGCAGAAT-3' (69.5°C), and

SEQ ID:NO:15 5'-GTGCAGCAGC AGAACAATTT GCTGAGGG-3' (72.1°C).

Other reagents and materials were obtained either from commercial sources or prepared using readily available starting materials and conventional procedures.

The following examples are included to illustrate the practice of this invention, and are not meant to be limiting in any way. All percentages are by weight unless otherwise indicated.

Example 1 Buffered Composition Containing hCMV DNA Primers

A preferred composition of this invention was prepared by mixing primers with additional PCR reagents. This composition contained tris(hydroxymethyl)aminomethane hydrochloride buffer (10 mmolar, pH 8), potassium chloride (50 mmolar), magnesium chloride (10 mmolar), gelatin (100 µg/ml),

dATP, dCTP, dGTP and dTTP (1.5 mmolar of each), glycerol (7.5%), primers (0.4 μmolar of each), DNA polymerase identified above (48 units/300 μl), and a monoclonal antibody specific to DNA polymerase identified above (50:1 molar ratio to DNA polymerase). The primers included were those identified as SEQ ID:NO:1-4 which are specific to nucleic acid sequences of hCMV DNA. The composition also contained phenol/chloroform purified CEM cells (normal uninfected lymphocytes, at either 2.75 or 6 μg/300 ml) to simulate a human blood sample.

Example 2 Simultaneous Amplification and Detection of HIV-I DNA and hCMV DNA

This example demonstrates the practice of the present invention using the composition described in Example 1 to simultaneously detect hCMV ("late antigen" region only) DNA along with HIV-I DNA, except that the composition further contained 0.4 μmolar of each of the primers identified above as SEQ ID:NO:10-13, and only primers SEQ ID:NO:3 and 4 for the "late antigen" region of hCMV DNA were used. The capture probes used were SEQ ID:NO:8, 14 and 15.

Twenty-four assays were carried out to detect the following various concentrations of the target nucleic acids in the test samples having two different amounts of CEM cells:

Sample a) 20,000 copies of hCMV DNA and 20,000 copies of HIV-I DNA,

Sample b) 500 copies of hCMV DNA and 500 copies of HIV-I DNA,

Sample c) 100 copies of hCMV DNA and 100 copies of HIV-I DNA,

Sample d) 100 copies of hCMV DNA and 20,000 copies of HIV-I DNA,

Sample e) 20,000 copies of hCMV DNA and 100 copies of HIV-I DNA, and

Sample f) 100 copies of hCMV DNA and 500 copies of HIV-I DNA.

In these assays, a nucleic acid sequence in the "late antigen" region of hCMV DNA was detected, and nucleic acid sequences in the "gag" and "env" regions of HIV-I DNA were detected. Two replicates were carried out for each assay.

The amplification and detection procedure for the assays were as follows:

Amplification:

Denature by heating at 95°C for 60 seconds,

40 cycles of priming and extending at 68°C for 30 seconds, and heating at 94°C for 15 seconds.

Detection:

Denature the amplified strands at 97°C for 120 seconds,

Capture the amplified products with the capture reagents at 50°C for 5 minutes,

Contact and incubate the captured products with the avidin-peroxidase conjugate solution at 40°C for 1 minute,

Wash the captured products using the wash solution at 40°C for 1 minute,

Add the dye-providing composition and incubate at 40°C for 2 minutes, and

Read the dye signal.

The results of the assays (two replicates of each assay) of Samples a)-f), are shown in the bar graphs of FIGS. 1-6, respectively, where the dye

signal is shown in the y-axis (where "0" represents no dye signal, and "10" represents highest dye density). In each figure, the first set of bar graphs are assays whereby 2.75 μg CEM cells were present, and the second set of bar graphs are assays whereby 6 μg CEM cells were present. Also, in all figures, the first bar (labeled as "1") in each set of bars represents the signal from hCMV DNA ("late antigen" region), the second bar (labeled as "2") represents the signal from HIV-I DNA ("gag" region), and the third bar (labeled as "3") represents the signal from HIV-I DNA ("env" region). The dye signals on both Control capture reagents were essentially zero, so they are not illustrated with bar graphs. In FIG.1, HIV-I DNA ("env" region) was not assayed at 6 μg CEM cells (there is no third bar, "3", for one set of data).

Example 3 Amplification and Detection of hCMV DNA Alone

This example was carried out similarly to Example 2 for the amplification and detection of hCMV DNA ("late antigen" region only) only in Samples a)-f) using the composition of Example 1 (2.75 μg CEM cells only).

FIG. 7 shows the dye signal results of the PCR process for the two replicates of each of Samples a)-c), and FIG. 8 shows the dye signal results of the PCR process for the replicates of each of Samples d)-f). Clear signals were observed for the presence of hCMV DNA (bar graphs labeled "1"). Small background signals were also observed (labeled as "2" and "3", respectively in each set of bar graphs) from the presence of HIV-I DNA ("gag" and "env" regions).

The invention has been described in detail with particular reference to preferred embodiments thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION

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(ii) TITLE OF THE INVENTION: DIAGNOSTIC COMPOSITIONS, ELEMENTS, METHODS AND TEST KITS FOR AMPLIFICATION AND DETECTION OF HUMAN CMV DNA USING PRIMERS HAVING MATCHED MELTING TEMPERATURES

(iii) NUMBER OF SEQUENCES: 26

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(F) ZIP: 1 4 6 5 0 - 2 2 0 1

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 MB storage (IBM)

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: MS-DOS Version 3.3

(D) SOFTWARE: PC-8 (Word for Windows)

(vi) CURRENT APPLICATION DATA:

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(vii) PRIOR APPLICATION DATE: None

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(2) INFORMATION FOR SEQ ID:NO:1
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for hCMV DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: U.S. 5,147,777
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:1

GAGGCTATTG TAGCCTACAC TTTGG 25

(3) INFORMATION FOR SEQ ID:NO:2
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for hCMV DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: U.S. 5,147,777
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:2

CAGCACCATC CTCCTCTTCC TCTGG 25

(5) INFORMATION FOR SEQ ID:NO:3

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Primer for hCMV DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:3

TGCACTGCCA GGTGCTTCGG CTCAT 25

(5) INFORMATION FOR SEQ ID:NO:4

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Primer for hCMV DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: U.S. 5,147,777

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:4

CACCACGCAG CGGCCCTTGA TGTTT 25

(6) INFORMATION FOR SEQ ID:NO:5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Probe for hCMV DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: U.S. 5,147,777

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:5

GGTGTCACCC CCAGAGTCCC CTGTACCCGC 30

(7) INFORMATION FOR SEQ ID:NO:6

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Probe for hCMV DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: U.S. 5,147,777

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:6

GACACAGTGT CCTCCCGCTC CTCCTGAGCA 30

(8) INFORMATION FOR SEQ ID:NO:7

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 nucleotides

(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Probe for hCMV DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: U.S. 5,147,777
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:7

GTGGAAGGCG GCTCGCTGGA AGCCGGTCGT 30

(9) INFORMATION FOR SEQ ID:NO:8
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Probe for hCMV DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: U.S. 5,147,777
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:8

GAACCGAGGG CCGGCTCACC TCTATGTTGG 30

(10) INFORMATION FOR SEQ ID:NO:9
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Probe for HIV-I DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:9

ATCCTGGGAT TAAATAAAAT AGTAAGAATG TATAGCCCTA C 41

(11) INFORMATION FOR SEQ ID:NO:10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Primer for HIV-I DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:10

AGTGGGGGGA CATCAAGCAG CCATGCAA 28

(12) INFORMATION FOR SEQ ID:NO:11

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Primer for HIV-I DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:11

CTTGGTTCTC TCATCTGGCC TGGTGC 26

(13) INFORMATION FOR SEQ ID:NO:12
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for HIV-I DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:12

TAGCACCCAC CAGGGCAAAG AGAAGAGT 28

(14) INFORMATION FOR SEQ ID:NO:13
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for HIV-I DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:13

AGATGCTGTT GCGCCTCAAT AGCCCTCA 28

(15) INFORMATION FOR SEQ ID:NO:14

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Probe for HIV-I DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:14

GAGACCATCA ATGAGGAAGC TGCAGAAT 28

(16) INFORMATION FOR SEQ ID:NO:15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Probe for HIV-I DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:15

GTGCAGCAGC AGAACAATTT GCTGAGGG 28

(17) INFORMATION FOR SEQ ID:NO:16

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Primer for hCMV DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:16

5'-CATTCCCACT GACTTTCTGA CGCACGT-3' 27

(18) INFORMATION FOR SEQ ID:NO:17

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 nucleotides

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Primer for hCMV DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:17

5'-TGAGGTCGTG GAACTTGATG GCGT-3' 24

(19) INFORMATION FOR SEQ ID:NO:18

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Probe for hCMV DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:18

5'-GGTCATCGCC GTAGTAGATG CGTAAGGCCT-3' 30

(20) INFORMATION FOR SEQ ID:NO:19
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for avian endogenous provirus ev-1 DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:19

5'-GGAATGACGC AAGGACATAT GGGCGT-3' 26

(21) INFORMATION FOR SEQ ID:NO:20
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 nucleotides

(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for avian endogenous provirus ev-1 DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:20

5'-CCCAGGTGCA CACCAATGTG GTGGAT-3' 26

(22) INFORMATION FOR SEQ ID:NO:21
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for positive control target DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:21

5'-GGACTGTGCG CGTTGTATAC CCTGC-3' 25

(23) INFORMATION FOR SEQ ID:NO:22
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 nucleotides

(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for positive control target DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:22

5'-ACTCCCGAAG CGAATGGCAC GTGGA-3' 25

(24) INFORMATION FOR SEQ ID:NO:23
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for positive control target DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:23

5'-CATAGCTTGT GCCCGTGTGG CACGT-3' 25

(25) INFORMATION FOR SEQ ID:NO:24
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 nucleotides
(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Primer for positive control target DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:24

5'-CCAAGACGAG ACCGTCAGAG CTGGT-3' 25

(26) INFORMATION FOR SEQ ID:NO:25
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Probe for avian endogenous provirus ev-1 DNA
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:25

5'-AAGCTGTTGC CGCCATCAAA TAAACG-3' 26

(27) INFORMATION FOR SEQ ID:NO:26
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Probe for positive control target DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID:NO:26

5'-CTGCGTTAGA CCGAGAACTG TGGATAAAGG-3' 30